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(54) Title: GENE ENCODING ALKALINE LIQUEFYING ALPHA-AMYLASE

(57) Abstract

The present invention provides a DNA fragment encoding alkaline liquefying α-amylase, recombinant DNA containing the DNA fragment, a transformed microorganism harboring the recombinant DNA, as well as a method for producing alkaline liquefying α-amylase using the transformant. The method of the present invention enables mass production of alkaline liquefying α-amylase useful as a detergent component.

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Description

Gene Encoding Alkaline Liquefying Alpha-Amylase

Technical Field:

The present invention relates to the gene encoding alkaline liquefying α-amylase and fragments thereof, and to recombinant DNA and a transformant bearing the gene or fragments of the gene.

Background Art:

Alpha-amylase has long been used in a variety of fields. For example, it has been used for the saccharification of grains and potatoes in the fermentation industry, as starch paste removers in the textile industry, as digestives in the pharmaceutical industry, and for the manufacture of thick malt syrups in the food industry. Alpha-amylase is an enzyme which acts on a starch-related polysaccharides such as amylose and amylopectin, hydrolyzing solely the α-1,4-glucoside bond of the polysaccharide molecule. Since 1833, when Payen and Persoz first discovered the enzyme, crystalline samples or electrophoretically homogeneous samples of α-amylase have been obtained from a number of different sources including bacteria, fungi, plant seeds, and animal digestive glands.

The present inventors have recently discovered that the efficacy of dish-washing detergents and laundry detergents for clothes can be greatly improved, particularly on starch

dirts, when α-amylase and a debranching enzyme are both incorporated into these detergents (Japanese Patent Application Laid-open (*kokai*) No. 2-132192). However, most of the α-amylases previously found in the natural world exhibit maximal and stable enzymatic activities in the neutral to acidic pH ranges, but scarcely work in an alkaline solution of pH 9-10. There exist only a small number of amylase enzymes that are known to exhibit maximal activities in the alkaline pH range (so-called alkaline α-amylases and alkali-resistant α-amylases). These alkaline α-amylases and alkali-resistant α-amylase include, an enzyme produced by *Bacillus* sp. A-40-2 [Horikoshi, K. *et al.*, Agric. Biol. Chem., 35, 1783 (1971)], an enzyme produced by *Bacillus* sp. NRRL B-3881 [Boyer, E., J. Bacteriol., 110, 992 (1972)], an enzyme produced by *Streptomyces* sp. KSM-9 (Japanese Patent Application Laid-Open (*kokai*) No. 61-209528, an enzyme produced by *Bacillus* sp. H-167 (Japanese Patent Application Laid-Open (*kokai*) No. 62-208278, an enzyme produced by *Bacillus alkalothermophilus* A3-8 (Japanese Patent Application Laid-Open (*kokai*) No. 2-49584, and an enzyme produced by *Natronococcus* sp. Ah-36 (Japanese Patent Application Laid-Open (*kokai*) No. 4-211369.

As used herein, the term "alkaline α-amylase" refers to α-amylases whose optimum pHs fall within the alkaline pH range, whereas the term "alkali-resistant α-amylase" refers to α-amylases which have optimum pHs within the neutral to acidic range but whose activities in the alkaline range are comparable with those obtained at an optimum pH, and in

addition, which retain their stabilities in the alkaline range. By the term "neutral range" is meant the range of pH not less than 6 and less than 8, and the term "alkaline" denotes a pH which is higher than the "neutral range".

Most of these alkaline α-amylases and alkali-resistant amylases are so-called saccharifying α-amylases which decompose starch or starch-related polysaccharides to glucose, maltose, or maltotriose. As such, these enzymes cause problems if they are used as enzymes for detergents, though they are advantageously used in the manufacture of sugar. Thus, there remains a need for so-called alkaline liquefying α-amylases which exhibit resistance against surfactants used in detergents, and which decompose starch or starch-related polysaccharides in a highly random manner. The present inventors continued an extensive search for microorganisms producing an alkaline liquefying α-amylase suitable as a detergent component, and they discovered that an alkalophilic *Bacillus* sp. KSM-AP1378 strain, having its optimum pH for growth in the alkaline range, produces an enzyme exhibiting the activity of an alkaline liquefying α-amylase. They elucidated that this enzyme is useful as an additive in detergent compositions for washing dishes and kitchen utensils and for detergent compositions for clothes (WO94/26881).

Amounts of the enzyme produced may be effectively increased by improving a method for culturing an alkaline liquefying α-amylase-producing microorganism, *Bacillus* sp. KSM-AP1378, or by exploiting mutation. However, in order to

produce the enzyme advantageously on an industrial scale, another approach must be taken.

Amounts of an enzyme produced can be enhanced using a genetic engineering approach, and in addition, the catalytic properties of the enzyme can be improved, using a protein engineering approach, by altering the gene encoding the enzyme. However, the gene encoding an alkaline liquefying α-amylase has not yet been obtained.

Accordingly, an object of the present invention is to provide the gene encoding alkaline liquefying α-amylase and fragments thereof, a transformant harboring recombinant DNA comprising the gene, and a method for producing an alkaline liquefying α-amylase using the transformant.

The DNA encoding the alkaline liquefying α-amylase gene may be further used to produce probes to be used in the isolation of additional, homologous alkaline liquefying α-amylase genes from other microorganisms. Thus, an additional object of the present invention is to provide a means of screening for and isolating additional alkaline liquefying α-amylase enzymes.

Disclosure of the Invention

The present inventors attempted to isolate, from the chromosomal DNA of an alkalophilic *Bacillus* strain, a DNA fragment containing the gene encoding an alkaline liquefying α-amylase, and as a result, they were successful in isolating an approximately 1.8 kb DNA fragment encoding an alkaline liquefying α-amylase. When they transformed a host microorganism using this DNA fragment ligated to a

suitable vector, it was confirmed that the resultant recombinant microorganism produced an alkaline liquefying α-amylase. Moreover, it was found that the amino acid sequence of the alkaline liquefying α-amylase to be encoded is different from that of previously known amylases. The present invention was accomplished based on this finding.

Accordingly, the present invention provides a DNA fragment encoding an alkaline liquefying α-amylase.

The present invention also provides a recombinant DNA comprising the above-described DNA fragment encoding an alkaline liquefying α-amylase.

The present invention also provides a transformed microorganism harboring the above-described recombinant DNA comprising a DNA fragment encoding an alkaline liquefying α-amylase.

The present invention further provides a method for producing an alkaline liquefying α-amylase, by culturing the above-described transformed microorganism and collecting the enzyme.

Brief Description of the Drawings

Fig. 1 shows a restriction enzyme map of a fragment of the gene encoding an alkaline liquefying amylase;

Fig. 2 is a chart depicting construction of pAML100 using a fragment of the gene encoding an alkaline liquefying amylase;

Fig. 3 shows nucleotide sequences of primers used.

Fig. 4 is a pH profile of an alkaline liquefying α-amylase produced by *Bacillus* sp. KSM-AP1378.

Best Mode for Carrying Out the Invention

In the present invention, a useful microorganism which serves as an alkaline liquefying α-amylase gene donor may be, for example, *Bacillus* sp. KSM-AP1378 (FERM BP-3048, deposited July 24, 1989 in Fermentation Research Institute, Agency of Industrial Science and Technology of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, 305 Japan), which is an alkalophilic *Bacillus* strain. This strain was isolated from the soil in the vicinity of the city of Tochigi in Tochigi Prefecture, Japan by the present inventors and identified as a strain which produces significant amounts of alkaline liquefying α-amylase. This strain was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, Japan) under FERM BP-3048 on August 8, 1990 (originally deposited as P-10886 on July 24, 1989).

In order to obtain chromosomal DNA from a donor microorganism, the method proposed by Marmur, J. (J. Mol. Biol., 3, 208 (1961)) or the method proposed by Saito, H. and Miura, K. (Biochem. Biophys. Acta, 72, 619 (1963)) may be used. Other similar methods may also be used.

DNA fragments comprising the alkaline liquefying α-amylase gene are prepared by cleaving the thus-obtained chromosomal DNA using restriction enzymes. Restriction enzymes which may be used are not particularly

limited so long as they do not fragment the gene. The alkaline liquefying α-amylase gene may also be obtained by PCR (Mullis, K.B. and Faloona, F.A., Methods Enzymol., 155, 335 (1987); Saiki, R. K. *et al.*, Science, 239, 487 (1988). For example, the gene may be obtained through the synthesis of primers having sequences corresponding to those on the upstream side of the 5'-terminus and on the downstream side of the 3'-terminus of the essential region based on the nucleotide sequence described in Sequence No. 2, and subsequently conducting PCR using, the chromosomal DNA of an alkaline liquefying α-amylase-producing microorganism as a template. Alternatively, an intact gene may be obtained by first obtaining a fragment of the alkaline liquefying α-amylase gene from an alkaline liquefying α-amylase-producing microorganism using any procedure, followed by PCR which amplifies the upstream and downstream sides of the fragmentary gene.

The thus-prepared genetic fragment is then subjected to cloning. Host/vector systems which may be used are not particularly limited, so far as that host bacterial strains express the alkaline liquefying α-amylase gene of the present invention, that the recombinant DNA molecules can be replicated in the host bacteria, and that the integrated gene can be stably harbored. For example, members of the EK system in which the host is *E. coli* K-12, and members of the BS system in which the host is *Bacillus subtilis* Marburg, may be used. Use of the EK system, which

encompasses many kinds of vectors and which is extensively studied genetically, provides good results and thus is preferred. Specific examples of host bacteria include HB101, C600, and JM109 of the EK system, and BD170, MI112, and ISW1214 of the BS system. Specific examples of vectors include pBR322 and pUC18 for the EK system, and pUB110 and pHY300PLK for the BS system.

A recombinant plasmid DNA molecule is created by cleaving a vector with a restriction enzyme followed by ligation with the above-mentioned chromosomal or PCR-amplified DNA fragment. The ligation may be achieved, for example, through the use of a DNA ligase.

Methods for transforming host bacterial strains using a recombinant DNA molecule are not particularly limited. For example, a calcium chloride method (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) may be used in the case of hosts of the EK system, and a protoplast method (Chang, C. and Cohen, S.N., Mol. Gen. Genet., 168, 111 (1978)) may be used in the case of hosts of the BS system. Selection of recombinant microorganisms are performed as follows. First, microorganisms which have been transformed with DNA which contains a vector-derived DNA fragment are selected, using as an index a character which is not inactivated by insertion of exogenous chromosomal DNA fragments, such as resistance to antibiotics coded onto the vector DNA. For example, in a specific case in which pBR322 of the EK system is used as a vector, and a *Hin*dIII fragment of chromosomal DNA is inserted into the *Hin*dIII cleavage

site of pBR322, the tetracycline resistant gene is inactivated, so a primary selection may be conducted by growth of the transformants that confer ampicillin resistance without having a *Hin*dIII cleavage site in the ampicillin gene. Subsequently, the selected transformants are transferred onto agar plates containing starch, using, for example, a replica method, and are then cultured so as to form colonies. By staining the starch contained in the starch-containing agar plates using an iodine-containing solution, target recombinant microorganisms can be selected as they decompose starch around the colonies.

The recombinant DNA molecule harbored by the thus-obtained recombinant microorganism can be extracted using standard procedures for preparing plasmids or phage DNAs (Maniatis, T. *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory, New York (1982)). When cleavage patterns obtained through the use of various restriction enzymes are analyzed by electrophoresis, it is confirmed that the recombinant DNA molecule is a ligated product of the vector DNA molecule and a DNA fragment containing the alkaline liquefying α-amylase gene.

The gene encoding an alkaline liquefying α-amylase is contained in a DNA fragment of about 2.1 kb shown in the restriction enzyme map of Fig. 1, and is present in the segment of about 1.6 kb shown by the white bar. The gene has a nucleotide sequence shown as Sequence No. 2. In this sequence, the 5' terminus and 3' terminus correspond to the left-hand side and the right-hand side, respectively, of

the fragment of about 2.1 kb shown as Sequence 2. In this sequence is observed an open reading frame (ORF) starting at the 145th nucleotide, ATG, and coding for a sequence consisting of 516 amino acid residues described in Sequence No. 1. Thirteen bases (13 b) upstream of the ORF, there exists a sequence AAGGAG which is highly complementary to the 3' terminal sequence of the 16S ribosomal RNA of *Bacillus subtilis* (McLaughlin, J.R. *et al.*, J. Biol. Chem., 256, 11283 (1981)). On a further upstream region extending nucleotides from 9 to 36, there exists a sequence TTGAAA ...... 16b ...... TATGGT which has high homology with the consensus sequence of a σ^A-type promoter (Gitt, M.A. *et al*, J. Biol. Chem., 260, 7178 (1985)). Similarly, another σ^A-type promoter sequence is found at nucleotides from 95 to 125. The amino acid sequence of the 10 amino acid residues on the amino terminus side in an alkaline liquefying α-amylase purified from a culture of *Bacillus* sp. KSM-AP1378 coincides with the sequence extending from the 37th amino acid (amino acid Nos. 37-46 in Sequence No. 2) deduced from the nucleotide sequence of the present DNA fragment.

When the nucleotide sequence of the gene of the present invention and a deduced amino acid sequence were compared with those of α-amylase known hitherto, it was confirmed that the present gene includes a novel nucleotide sequenced, with the deduced amino acid sequence encoded by the gene being different from those of other α-amylases such as a liquefying α-amylase produced by *Bacillus amylolique* (Takkinen, K. *et al.*, J. Biol. Chem., 258, 1007 (1983)), a liquefying α-amylase

produced by *Bacillus stearothermophilus* (Nakajima, R. *et al.*, J. Bacteriol., 163, 401 (1985)), a liquefying α-amylase produced by *Bacillus licheniformis* (Yuuki, T *et al.*, J. Biochem., 98, 1147 (1985)), or a liquefying α-amylase produced by *Bacillus* sp. 707 (Tsukamoto, A. *et al.*, Biochem. Biophys. Res. Commun., 151, 25 (1988)).

An example of a preferred recombinant DNA molecule containing the entire region of the alkaline liquefying α-amylase gene is plasmid pAML100 (Fig. 2). This recombinant plasmid has a size of 4.4 kb and formed of a fragment containing a 1.8 kb fragment which contains the alkaline liquefying α-amylase gene and pUC19. An example of a preferred recombinant microorganism harboring the recombinant DNA molecule is an *E. coli* HB101(pAML100) strain. This strain was obtained by transforming *E. coli* HB101 strain with the recombinant plasmid pAML100 using a standard transformation method. When this strain is cultured using a medium routinely employed for culturing *E. coli,* it produces an alkaline liquefying α-amylase. The optimum reaction pH of the thus-produced enzyme is pH 8-9. This agrees well with the activity-pH relationship profile determined for the alkaline liquefying α-amylase produced by the gene donor bacterial strain, *Bacillus* sp. KSM-AP1378 (Fig. 4).

The DNA fragments of the present invention are not necessarily limited only to those encoding the amino acid sequences shown in the below-described sequence listing, so far as they encode a protein exhibiting the enzymatic activity of interest, and they encompass DNA fragments

encoding an amino acid sequence in which one or more amino acids are substituted, added, deleted, inverted, or inserted. An example of such DNA is one encoding an amino acid sequence equivalent to the amino acid sequence described in Sequence No. 1 from which up to 32 amino acids on the N-terminal side have been deleted.

In order to produce an alkaline liquefying α-amylase using the transformed microorganism of the present invention, a transformed microorganism harboring the aforementioned DNA fragment of the present invention is subjected to culturing. Alternatively, the DNA fragment may be integrated in a variety of expression vectors to obtain transformed microorganisms with enhanced expression ability, followed by culturing of the resultant transformants. Moreover, the transformed microorganisms may be cultured under different conditions depending on the identity of the microorganisms. Thus, culture conditions suited for the host may be used.
In order to collect an alkaline liquefying α-amylase from the resultant culture, a routine method (such as the method described in WO94/26881) may be used.

The DNA fragments of the present invention may be further used as probes for the isolation of homologous alkaline liquefying α-amylase genes from other organisms.

Examples

The present invention will next be described in more detail by way of examples, which should not be construed as limiting the invention thereto. Concentrations in the

Examples are all on a basis of % by weight.

Example 1:

Bacillus sp. KSM-AP1378 producing an alkaline liquefying α-amylase was inoculated in 5 ml of medium A (Table 1) and subjected to shaking culture at 30°C for 24 hours. One ml of the culture was inoculated in 100 ml of the same medium, followed by shaking culture at 30°C for a further 12 hours. Subsequently, cells were collected by centrifugation and about 1 mg of chromosomal DNA was obtained in accordance with a method proposed by Saito and Miura (Saito, H. and Miura K., Biochim Biophys. Acta, 72, 619 (1963)).

Table 1

Composition of medium A

Soluble starch	1.0%
Polypepton	1.0%
Yeast extract	0.5%
KH_2PO_4	0.1%
$Na_2HPO_4 \cdot 12H_2O$	0.25%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 2H_2O$	0.02%
$FeSO_4 \cdot 7H_2O$	0.001%
$MnCl_2 \cdot 4H_2O$	0.0001%
Na_2CO_3	1.0% (separately sterilized)

Example 2:

It is known that many members of the amylase family

possess I-IV regions where amino acid sequences are conserved at a high level (Nakajima, R. *et al.*, Appl. Microbiol. Biotechnol., 23, 355 (1986)). Therefore, primers 1 and 2 (Figs. 1 and 3) corresponding to regions II and IV were synthesized based on the amino acid sequence of region II and the amino acid sequence of region IV, which are particularly conserved regions among regions I through IV of known alkaline liquefying α-amylases. Using the thus-synthesized primers and chromosomal DNA of KSM-AP1378 (which served as template), PCR was conducted (one cycle = 94°C x 1 min. + 42°C x 1 min. + 60°C x 2 min., 30 cycles). A gene fragment of approximately 0.3 kb (fragment A) shown in Fig. 1 was obtained, and the nucleotide sequence of this fragment was determined. As a result, it was found that the present fragment was coded with an amino acid sequence exhibiting a non-negligible level of homology with the amino acid sequence extending from region II through region IV of known liquefying amylase.

Example 3:

Using fragment A as a probe, chromosomal DNA of *Xba*I-digested KSM-AP1378 was subjected to Southern hybridization. As a result, it was confirmed that there was a band which hybridized at the location of approximately 1.0 kb. An amplified fragment of approximately 0.7 kb (fragment B) was obtained by an inverse PCR method (Triglia, T. *et al.*, Nucleic Acids Res., 16, 81 (1988)) using primers synthesized from the terminal sequences of fragment A (on the

side of region II: primer 3; on the side of region IV: primer 4) and DNAs which had been obtained by intramolecularly ligating *Xba*I-digested KSM-AP1378 chromosomal DNA (Fig. 1) as template. The nucleotide sequence of fragment B was determined, which revealed that the present fragment contained a stretch, approximately 0.6 kb region downstream from region IV. The present fragment contained a termination codon for the ORF, which was deduced to be attributed to alkaline liquefying α-amylase.

Example 4:

A primer was designed and synthesized based on the N-terminal amino acid sequence (7 amino acids) of alkaline liquefying α-amylase from the KSM-AP1378 strain (Fig. 3). Using the resultant primer (primer 5) in combination with the aforementioned primer 3 (Fig. 3) and, as a template, chromosomal DNA of KSM-AP1378, PCR was conducted to obtain a fragment of approximately 0.7 kb (fragment C, Fig. 1), thereby determining its nucleotide sequence.

Example 5:

A primer containing 21 bases, stretching directly downstream of the nucleotide sequence encoding N-terminal amino acid sequence of the purified enzyme, was synthesized (primer 6). Using primers 6 and 7 (Figs. 1 and 3) and DNAs which had been obtained by intramolecularly ligating *Hin*dIII-digested KSM-AP1378 chromosomal DNA (Fig. 1) as templates,

an inverse PCR method was performed, obtaining a 1.2 kb fragment in which an upstream 0.8 kb fragment (fragment D) and a downstream *Pst*I-*Hin*dIII 0.4 kb fragment had been ligated at the *Hin*dIII site. The nucleotide sequence of the fragment D region was determined, which revealed the presence of a signal sequence composed of 31 amino acids, MKLHNRIISVLLTLLLAVAVLFPYMTEPAQA (from No. 1 to No. 31 of Sequence No. 2), a deduced SD sequence composed of AAGGAG (nucleotides 127-132; McLaughlin, J.R. *et al.*, J. Biol. Chem., 260, 7178 (1985)), and two kinds of deduced promoter sequences (-35 sequences, TTGAAA; -10 sequence, TATGGT, and -35 sequence, TTGACT; -10 sequence, TAAATT).

Example 6:

Using primer A located at approximately 0.1 kb upstream of the promoter sequence, primer B located 79 b downstream of the termination codon, and chromosomal DNA of KSM-AP1378 as templates, a stretch of approximately 1.8 kb between the primers was amplified by PCR. The resultant amplified fragment was inserted into the *Sma*I site of pUC19, and then introduced into *E. coli* HB101. The transformant was allowed to grow on an LB agar medium containing 0.4% Starch azure and 15 µg/ml ampicillin. Colonies which had formed transparent halos around them were isolated as an *E. coli* strain that produced liquefying α-amylase. A recombinant plasmid was prepared from this transformant, and a restriction enzyme map of the plasmid was made. In the map, it was confirmed that an approximately 1.8 kb DNA fragment (fragment E) shown in Fig. 1 was contained. This recombinant plasmid was designated plasmid

pAML100 (Fig. 2).

Example 7:

The recombinant *E. coli* obtained in Example 6 was subjected to shaking culture for 12 hours in 5 ml of an LB liquid medium containing 50 µg/ml of ampicillin. One (1) ml of the culture was inoculated to 100 ml of an LB medium (containing ampicillin), followed by shaking culture at 37°C for 24 hours. Cells collected by centrifugal separation were suspended in Tris-HCl buffer (pH 8.0), and were disrupted by sonication. After the cells were sonicated, cell debris was removed by centrifugal separation, and the resultant supernatant was used as a cell-free extract. As a control, the cell-free extract of HB101(PUC19) strain was separately prepared in a similar manner. α-Amylase activities in these extracts were measured by first causing a reaction, at 50°C for 15 minutes, in a reaction mixture containing 50 mM glycine-NaCl-NaOH buffer (pH 10) and soluble starch, and then by quantitatively determining the produced reducing sugar by the 3,5-dinitrosalicylic acid method (WO94/26881). One unit of enzymatic activity was defined as the amount of protein that produced a quantity per minute of reducing sugar equivalent to 1 µmol of glucose. As a result, α-amylase activity was detected in the cell-free extract of strain HB101(pAML100). The optimum working pH of α-amylase was found to fall within the pH range between 8 and 9. This result coincides well with the optimum pH of liquefying α-amylase produced by *Bacillus* sp. KSM-AP1378

(Fig. 4). For the measurement of enzymatic activities, the buffers shown in Table 2 below were used (each at 40 mM).

Table 2

pH 3.5-5.5:	Acetate buffer
pH 5.5-8.5:	Tris-maleic acid buffer
pH 8.5-10.5:	Glycine-NaCl-NaOH buffer
pH 10.5-11.0:	Na_2CO_3-$NaHCO_3$ buffer

Industrial Applicability:

According to the present invention, it is possible to obtain a gene encoding for alkaline liquefying α-amylase exhibiting the maximum activity in the alkaline pH range as well as a microorganism harboring such gene. Use of them facilitates mass production of alkaline liquefying α-amylase.

Sequence Listing

Information for Sequence No. 1:

(i) Sequence Characteristics:

(A) Length: 516 amino acids

(B) Type: amino acid

(D) Topology: linear

(ii) Molecule Type: peptide

(xi) Sequence Description: Sequence No. 1:

Met Lys Leu His Asn Arg Ile Ile Ser Val Leu Leu Thr Leu Leu Leu
1 5 10 15

Ala Val Ala Val Leu Phe Pro Tyr Met Thr Glu Pro Ala Gln Ala His
20 25 30

His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His Leu
35 40 45

Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala Asn
50 55 60

Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp Lys
65 70 75 80

Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp
85 90 95

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
100 105 110

Arg Ser Gln Leu Gln Gly Ala Val Thr Ser Leu Lys Asn Asn Gly Ile
115 120 125

Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp Gly
130 135 140

Thr Glu Met Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn Gln
145 150 155 160

Glu Ile Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp Phe
165 170 175
Pro Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr His
180 185 190
Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys Ile
195 200 205
Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp Ile
210 215 220
Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met Asp
225 230 235 240
His Pro Glu Val Ile Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr Thr
245 250 255
Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His Ile
260 265 270
Lys Tyr Ser Tyr Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr Thr
275 280 285
Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu Ala
290 295 300
Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val Phe
305 310 315 320
Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly Gly
325 330 335
Tyr Phe Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys His
340 345 350
Pro Ile His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro Gly
355 360 365
Glu Ala Leu Glu Ser Phe Val Gln Ser Trp Phe Lys Pro Leu Ala Tyr
370 375 380

Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr Gly
385 390 395 400
Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ser Met Lys Ser Lys
405 410 415
Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Tyr Ala Tyr Gly Thr Gln
420 425 430
His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu Gly
435 440 445
Asp Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp Gly
450 455 460
Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys His Lys Ala Gly Gln
465 470 475 480
Val Trp Arg Asp Ile Thr Gly Asn Arg Ser Gly Thr Val Thr Ile Asn
485 490 495
Ala Asp Gly Trp Gly Asn Phe Thr Val Asn Gly Gly Ala Val Ser Val
500 505 510
Trp Val Lys Gln
516

Information for Sequence No. 2:

(i) Sequence Characteristics:

(A) Length: 1776 base pairs

(B) Type: nucleic acid

(C) Strandedness: double

(D) Topology: linear

(ii) Molecule Type: DNA (genomic)

(vi) Original Source:

(A) Organism: *Bacillus* sp.

(B) Strain: KSM-AP1378

(xi) Sequence Description: Sequence No. 2:

```
ATATAAATTT GAAATGAACA CCTATGAAAA TATGGTAGCG ATTGCGCGAC GAGAAAAAAC     60
TTGGGAGTTA GGAAGTGATA TTAAAGGATT TTTTTTGACT TGTTGTGAAA ACGCTTGCAT    120
AAATTGAAGG AGAGGGTGCT TTTT ATG AAA CTT CAT AAC CGT ATA ATT AGC GTA   174
                           Met Lys Leu His Asn Arg Ile Ile Ser Val
                            1               5                  10
CTA TTA ACA CTA TTG TTA GCT GTA GCT GTT TTG TTT CCA TAT ATG ACG      222
Leu Leu Thr Leu Leu Leu Ala Val Ala Val Leu Phe Pro Tyr Met Thr
            15                  20                  25
GAA CCA GCA CAA GCC CAT CAT AAT GGG ACG AAT GGG ACC ATG ATG CAG      270
Glu Pro Ala Gln Ala His His Asn Gly Thr Asn Gly Thr Met Met Gln
        30                  35                  40
TAT TTT GAA TGG CAT TTG CCA AAT GAC GGG AAC CAC TGG AAC AGG TTA      318
Tyr Phe Glu Trp His Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu
    45                  50                  55
CGA GAT GAC GCA GCT AAC TTA AAG AGT AAA GGG ATT ACC GCT GTT TGG      366
Arg Asp Asp Ala Ala Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp
60                  65                  70
ATT CCT CCT GCA TGG AAG GGG ACT TCG CAA AAT GAT GTT GGG TAT GGT      414
Ile Pro Pro Ala Trp Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly
75                  80                  85                  90
GCC TAT GAT TTG TAC GAT CTT GGT GAG TTT AAC CAA AAG GGA ACC GTC      462
Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val
                95                  100                 105
CGT ACA AAA TAT GGC ACA AGG AGT CAG TTG CAA GGT GCC GTG ACA TCT      510
```

110 115 120

TTG AAA AAT AAC GGG ATT CAA GTT TAT GGG GAT GTC GTG ATG AAT CAT 558

Leu Lys Asn Asn Gly Ile Gln Val Tyr Gly Asp Val Val Met Asn His

125 130 135

AAA GGT GGA GCA GAC GGG ACA GAG ATG GTA AAT GCG GTG GAA GTG AAC 606

Lys Gly Gly Ala Asp Gly Thr Glu Met Val Asn Ala Val Glu Val Asn

140 145 150

CGA AGC AAC CGA AAC CAA GAA ATA TCA GGT GAA TAC ACC ATT GAA GCA 654

Arg Ser Asn Arg Asn Gln Glu Ile Ser Gly Glu Tyr Thr Ile Glu Ala

155 160 165 170

TGG ACG AAA TTT GAT TTC CCT GGA AGA GGA AAT ACC CAT TCC AAC TTT 702

Trp Thr Lys Phe Asp Phe Pro Gly Arg Gly Asn Thr His Ser Asn Phe

175 180 185

AAA TGG CGC TGG TAT CAT TTT GAT GGG ACA GAT TGG GAT CAG TCA CGT 750

Lys Trp Arg Trp Tyr His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg

190 195 200

CAG CTT CAG AAC AAA ATA TAT AAA TTC AGA GGT ACC GGA AAG GCA TGG 798

Gln Leu Gln Asn Lys Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp

205 210 215

GAC TGG GAA GTA GAT ATA GAG AAC GGC AAC TAT GAT TAC CTT ATG TAT 846

Asp Trp Glu Val Asp Ile Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr

220 225 230

GCA GAC ATT GAT ATG GAT CAT CCA GAA GTA ATC AAT GAA CTT AGA AAT 894

Ala Asp Ile Asp Met Asp His Pro Glu Val Ile Asn Glu Leu Arg Asn

235 240 245 250

TGG GGA GTT TGG TAT ACA AAT ACA CTT AAT CTA GAT GGA TTT AGA ATC 942

Trp Gly Val Trp Tyr Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile

255 260 265

GAT GCT GTG AAA CAT ATT AAA TAC AGC TAT ACG AGA GAT TGG CTA ACA 990

Asp Ala Val Lys His Ile Lys Tyr Ser Tyr Thr Arg Asp Trp Leu Thr

270 275 280

CAT GTG CGT AAC ACC ACA GGT AAA CCA ATG TTT GCA GTT GCA GAA TTT 1038

His Val Arg Asn Thr Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe

285 290 295

TGG AAA AAT GAC CTT GCT GCA ATC GAA AAC TAT TTA AAT AAA ACA AGT 1086

Trp Lys Asn Asp Leu Ala Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser

300 305 310

TGG AAT CAC TCC GTG TTC GAT GTT CCT CTT CAT TAT AAT TTG TAC AAT 1134

Trp Asn His Ser Val Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn

315 320 325 330

GCA TCT AAT AGT GGT GGC TAT TTT GAT ATG AGA AAT ATT TTA AAT GGT 1182

Ala Ser Asn Ser Gly Gly Tyr Phe Asp Met Arg Asn Ile Leu Asn Gly

335 340 345

TCT GTC GTA CAA AAA CAC CCT ATA CAT GCA GTC ACA TTT GTT GAT AAC 1230

Ser Val Val Gln Lys His Pro Ile His Ala Val Thr Phe Val Asp Asn

350 355 360

CAT GAC TCT CAG CCA GGA GAA GCA TTG GAA TCC TTT GTT CAA TCG TGG 1278

His Asp Ser Gln Pro Gly Glu Ala Leu Glu Ser Phe Val Gln Ser Trp

365 370 375

TTC AAA CCA CTG GCA TAT GCA TTG ATT CTG ACA AGG GAG CAA GGT TAC 1326

Phe Lys Pro Leu Ala Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr

380 385 390

CCT TCC GTA TTT TAC GGT GAT TAC TAC GGT ATA CCA ACT CAT GGT GTT 1374

Pro Ser Val Phe Tyr Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val

395 400 405 410

CCT TCG ATG AAA TCT AAA ATT GAT CCA CTT CTG CAG GCA CGT CAA ACG 1422

Pro Ser Met Lys Ser Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr

415 420 425

TAT GCC TAC GGA ACC CAA CAT GAT TAT TTT GAT CAT CAT GAT ATT ATC 1470

Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe Asp His His Asp Ile Ile

430 435 440

GGC TGG ACG AGA GAA GGG GAC AGC TCC CAC CCA AAT TCA GGA CTT GCA 1518

Gly Trp Thr Arg Glu Gly Asp Ser Ser His Pro Asn Ser Gly Leu Ala

445 450 455

ACT ATT ATG TCC GAT GGG CCA GGG GGT AAT AAA TGG ATG TAT GTC GGG 1566

Thr Ile Met Ser Asp Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly

460 465 470

AAA CAT AAA GCT GGC CAA GTA TGG AGA GAT ATC ACC GGA AAT AGG TCT 1614

Lys His Lys Ala Gly Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Ser

475 480 485 490

GGT ACC GTC ACC ATT AAT GCA GAT GGT TGG GGG AAT TTC ACT GTA AAC 1662

Gly Thr Val Thr Ile Asn Ala Asp Gly Trp Gly Asn Phe Thr Val Asn

495 500 505

GGA GGG GCA GTT TCG GTT TGG GTG AAG CAA TAAATAAGGA ACAAGAGGCG 1712

Gly Gly Ala Val Ser Val Trp Val Lys Gln

510 515

AAAATTACTT TCCTACATGC AGAGCTTTCC GATCACTCAT ACACCCAATA TAAATTGGAA 1772

GCTT 1776

CLAIMS:

1. A DNA molecule encoding alkaline liquefying α-amylase activity.

2. A DNA molecule as defined in Claim 1, which encodes the amino acid sequence described in Sequence No. 1 or a functional fragment thereof.

3. A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity and possessing an amino acid sequence described in Sequence No. 1 in which one or more amino acids are substituted, added, deleted, inverted, or inserted.

4. A DNA molefule as defined in any one of Claims 1 through 3, further comprising a nucleotide sequence for regulating expression of a gene.

5. A recombinant DNA containing the DNA molecule of any one of Claims 1 through 4.

6. A transformed microorganism harboring the recombinant DNA of Claim 5.

7. A method for producing alkaline liquefying α-amylase, comprising culturing the transformed microorganism of Claim 6 and isolating the alkaline liquefying α-amylase produced by the microorganism.

8. A DNA molecule which hybridizes to a DNA sequence which is complementary to the nucleic acid sequence of SEQ ID No. 2.

9. A protein encoded by the DNA molecule of Claim 9.

10. A DNA molecule which hybridizes to a DNA sequence which is complementary to the nucleic acid sequence of SEQ ID No. 2, wherein said DNA molecule encodes a protein having alkaline

liquefying α-amylase activity.

11. A protein encoded by the DNA molecule of Claim 11.

12. The recombinant DNA plasmid pAML100.

13. The recombinant *E. coli* strain HB101(pAML100).

The present invention provides a DNA fragment encoding alkaline liquefying α-amylase, recombinant DNA containing the DNA fragment, a transformed microorganism harboring the recombinant DNA, as well as a method for producing alkaline liquefying α-amylase using the transformant. The method of the present invention enables mass production of alkaline liquefying α-amylase useful as a detergent component.

AMENDED CLAIMS

[received by the International Bureau on 11 December 1996 (11.12.96); original claims 4, 9, 11 amended; remaining claims unchanged (2 pages)]

1. A DNA molecule encoding alkaline liquefying α-amylase activity.

2. A DNA molecule as defined in Claim 1, which encodes the amino acid sequence described in Sequence No. 1 or a functional fragment thereof.

3. A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity and possessing an amino acid sequence described in Sequence No. 1 in which one or more amino acids are substituted, added, deleted, inverted, or inserted.

4. A DNA molecule as defined in any one of Claims 1 through 3, further comprising a nucleotide sequence for regulating expression of a gene.

5. A recombinant DNA containing the DNA molecule of any one of Claims 1 through 4.

6. A transformed microorganism harboring the recombinant DNA of Claim 5.

7. A method for producing alkaline liquefying α-amylase, comprising culturing the transformed microorganism of Claim 6 and isolating the alkaline liquefying α-amylase produced by the microorganism.

8. A DNA molecule which hybridizes to a DNA sequence which is complementary to the nucleic acid sequence of SEQ ID No. 2.

9. A protein encoded by the DNA molecule of Claims 1 through 4.

10. A DNA molecule which hybridizes to a DNA sequence which is complementary to the nucleic acid sequence of SEQ ID No. 2, wherein said DNA molecule encodes a protein having alkaline

liquefying α-amylase activity.

11. A protein encoded by the DNA molecule of Claim 10.

12. The recombinant DNA plasmid pAML100.

13. The recombinant *E. coli* strain HB101(pAML100).

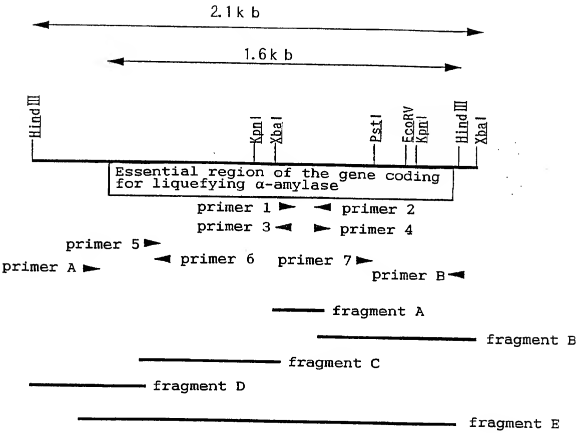

2.1 k b
1.6 k b
HindIII
KpnI
XbaI
PstI
EcoRV
KpnI
HindIII
XbaI
Essential region of the gene coding for liquefying α-amylase
primer 1
primer 2
primer 3
primer 4
primer 5
primer 6
primer 7
primer A
primer B
fragment A
fragment B
fragment C
fragment D
fragment E

FIG. 1

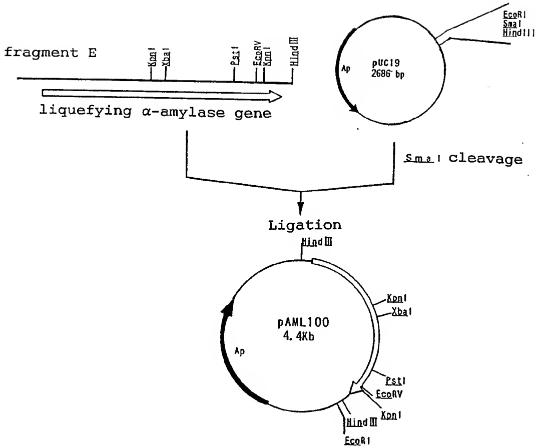
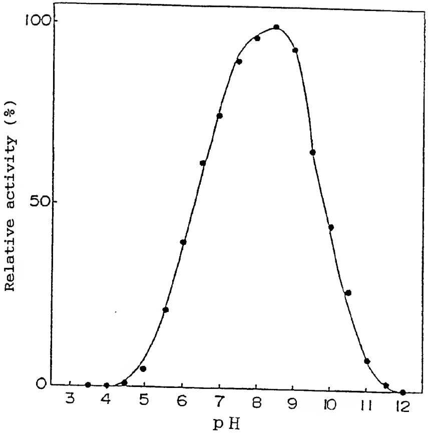

fragment E
KpnI
XbaI
PstI
EcoRV
KpnI
HindIII
liquefying α-amylase gene
EcoRI
SmaI
HindIII
Ap
pUC19
2686 bp
SmaI cleavage
Ligation
HindIII
KpnI
XbaI
pAML100
4.4Kb
Ap
PstI
EcoRV
KpnI
HindIII
EcoRI

FIG. 2

FIG. 3

```
primer 1   5' TAGACGCAGTAAAACACATAAA 3'
               C  T  C  C  G  T  C
               G     G  G        T
               T     T  T

primer 2   3' CGACAATGAAAACAACTATTAGTACT 5'
                G  G  G  G  G  G  G  G
                C  C  C     C
                T  T  T     T

primer 3   5' AGCCAATCTCTCGTATAGCTGTA 3'

primer 4   5' GTACAAAAACACCCTATACATG 3'

primer 5   5' AATGGAACAATGATGCAGTA 3'
                   T  T        T

primer 6   5' CATTTGGCAAATGCCATTCAAA 3'

primer 7   5' AAAATTGATCCACTTCTGCAG 3'

primer A   5' CAGCGCGTGATAATATAAATTTGAAT 3'

primer B   5' AAGCTTCCAATTTATATTGGGTGTAT 3'
```

FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 96/01641

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N9/28 C12N1/21 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 26881 (KAO CORP ;ARA KATSUTOSHI (JP); SAEKI KATSUHISA (JP); IGARASHI KAZU) 24 November 1994 cited in the application see the whole document & EP,A,0 670 367 (KAO CORPORATION) ---	1-13
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 151, no. 1, 29 February 1988, pages 25-31, XP000605386 TSUKAMOTO A. ET AL.: "Nucleotide sequence of the maltohexaose-producing amylase gene from alkalophilic Bacillus sp. #707 and structural similarity to liquefying type alpha-amylases."	1,3-11
A	see the whole document --- -/--	2,12,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
6 November 1996	15. 11. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Mandl, B

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Inter   nal Application No
PCT/JP 96/01641

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOCHEMISTRY, vol. 98, no. 5, 1985, pages 1147-1156, XP002017641 YUUKI T ET AL.: "Complete nucleotide sequence of a gene coding for heat- and pH-stable alpha-amylase of Bacillus licheniformis: Comparison of the amino acid sequences of three bacterial liquefying alpha-amylases deduced from the DNA sequences."	1,3-11
A	see the whole document	2,12,13
A	EP,A,0 410 498 (GIST BROCADES NV ;PLANT GENETIC SYSTEMS NV (BE)) 30 January 1991 see the whole document	3
P,X	WO,A,95 26397 (NOVONORDISK AS ;OUTTRUP HELLE (DK); BISGAARD FRANTZEN HENRIK (DK);) 5 October 1995 see the whole document	1,3-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/JP 96/01641

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9426881	24-11-94	CN-A-	1110058	11-10-95
		EP-A-	0670367	06-09-95
EP-A-0410498	30-01-91	AU-B-	638263	24-06-93
		AU-A-	5953890	17-01-91
		CA-A-	2030554	30-12-90
		CN-A-	1050220	27-03-91
		WO-A-	9100353	10-01-91
		JP-T-	4500756	13-02-92
		US-A-	5364782	15-11-94
WO-A-9526397	05-10-95	AU-A-	2067795	17-10-95
		ZA-A-	9502565	21-12-95